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Paramyxovirus Assembly and Budding: Building Particles that Transmit Infections

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Abstract

The paramyxoviruses define a diverse group of enveloped RNA viruses that includes a number of important human and animal pathogens. Examples include human respiratory syncytial virus and the human parainfluenza viruses, which cause respiratory illnesses in young children and the elderly; measles and mumps viruses, which have caused recent resurgences of disease in developed countries; the zoonotic Hendra and Nipah viruses, which have caused several outbreaks of fatal disease in Australia and Asia; and Newcastle disease virus, which infects chickens and other avian species. Like other enveloped viruses, paramyxoviruses form particles that assemble and bud from cellular membranes, allowing the transmission of infections to new cells and hosts. Here, we review recent advances that have improved our understanding of events involved in paramyxovirus particle formation. Contributions of viral matrix proteins, glycoproteins, nucleocapsid proteins, and accessory proteins to particle formation are discussed, as well as the importance of host factor recruitment for efficient virus budding. Trafficking of viral structural components within infected cells is described, together with mechanisms that allow for the selection of specific sites on cellular membranes for the coalescence of viral proteins in preparation of bud formation and virion release.

Keywords

virus budding; virus assembly; matrix protein; paramyxovirus; virus-like particle; polarized budding; lipid raft membranes; viral trafficking

1. Introduction

The paramyxoviruses define a large group of enveloped RNA viruses, some of which cause significant human and animal diseases. Examples include human respiratory syncytial virus (HRSV), human parainfluenza virus types 1-4 (hPIV 1-4), measles virus, mumps virus, Nipah virus, Hendra virus, and Newcastle disease virus (NDV). HRSV and hPIV types 1–3 are major contributors to respiratory infections in young children and the elderly (Counihan

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et al., 2001; Falsey, 1998; Hall et al., 2009; Heilman, 1990; Welliver, 2003). HRSV infection is the leading cause of severe pediatric respiratory tract disease, causing an estimated 64 million cases and 160,000 annual deaths globally (Hall et al., 2009; Welliver, 2003; WHO, 2009a; Wright et al., 2005; Zhang et al., 2002). There are currently no effective vaccines to prevent HRSV or hPIV infections. Although both measles virus and mumps virus infections are vaccine-preventable, these infections remain a health burden in developing countries, and several significant outbreaks attributed to low vaccination rates have occurred recently in the United Kingdom, Canada, and the United States (CDC, 2006a; CDC, 2006b; CDC, 2008; Dayan et al., 2008; Hviid et al., 2008; Peltola et al., 2007). Nipah virus and Hendra virus (Henipaviruses) cause deadly infections in humans, with severe and widespread vasculitis and encephalitis resulting in a mortality rate of about 40% (Bishop and Broder, 2008). The viruses are zoonotic, and the natural hosts are fruit bats, such as flying foxes. The viruses spread to humans mainly through intermediary hosts: horses in the case of Hendra virus, and pigs in the case of Nipah virus (Eaton et al., 2006). Twelve recognized outbreaks of Nipah virus infection in South Asia have occurred since its identification in 1999 (Epstein et al., 2006; Gurley et al., 2007; WHO, 2009) and a total of thirteen known outbreaks of Hendra virus in Australia have occurred, the first of which were recognized in 1994 (Bishop and Broder, 2008; ProMED-mail, 2009). There are currently no effective treatments or vaccines approved for Henipavirus infections, and outbreaks are likely to continue so long as humans and their domesticated animals encroach into geographic locations occupied by flying foxes. Such outbreaks have potential agricultural significance as well; the initial Malaysian epidemic caused by Nipah virus was contained only after 1.1 million pigs were culled (Mohd Nor et al., 2000). Other paramyxoviruses of agricultural importance include NDV, which causes a highly contagious respiratory and neurological disease in many avian species, including chickens (Alexander, 2009) and rinderpest virus, which causes disease in cattle (Roeder and Taylor, 2002). Additional viruses that have been widely used as laboratory models for the study of paramyxovirus entry and exit include parainfluenza virus 5 (PIV5, formerly SV5) and Sendai virus.

Virus particles are containers built within infected cells that are meant to transmit infection from cell-to-cell and from host-to-host. Enveloped virus particles form by budding from cellular membranes. Buds emerge from selected sites on the membranes where viral proteins and genomes have assembled together, then pinch off to achieve particle release (Fig. 1). The resulting virions have outer surfaces that consist of host-derived membrane, enriched with viral integral membrane glycoproteins (Fig. 2). The paramyxoviruses encode two glycoproteins: a fusion (F) protein, and an attachment [HN (hemagglutinin-neuraminidase), H (hemagglutinin), or G (glyco-)] protein. These proteins are packed very densely into the viral envelopes, forming “spike” layers that are visible by electron microscopy (Fig. 2B). Enclosed within paramyxovirus particles are the RNA genomes, bound with nucleocapsid (N or NP) proteins to form helical structures called ribonucleoproteins (RNPs). Directly underlying the viral membranes are the viral matrix (M) proteins, which bridge the viral glycoproteins and RNPs, thereby organizing virus assembly. Paramyxoviruses form particles that are mainly spherical, but sometimes filamentous, with considerable variation in size and shape. Particles typically range in size from 150 to 300 nm in diameter, but can reach diameters of greater than 1 μm in some cases (Goldsmith et al., 2003).

Paramyxovirus infections (see Fig. 3) are initiated when virus particles bind to receptor molecules on the surfaces of target cells. For some paramyxoviruses, such as Sendai virus and mumps virus, attachment is mediated by HN proteins that bind to sialic acid receptors. These HN proteins also possess sialidase activities, which function later in the virus lifecycle to facilitate the separation of virions from infected cells and to prevent virion aggregation. Other paramyxovirus attachment proteins mediate binding to protein receptors. These include the H protein of measles virus and the G proteins of the Henipaviruses.

Following virion attachment, viral F proteins are triggered, resulting in the fusion of virion membranes with target cell membranes via a process that is driven by the refolding of F proteins from initial metastable states into more stable hairpin structures (reviewed in Lamb and Parks, 2006;Russell and Luque, 2006). Completion of this process allows virion contents, including RNPs, to enter the cytoplasms of target cells. Viral transcription then occurs, with negative-sense viral genomic RNA within RNPs serving as templates for the production of mRNAs by viral RNA-dependent RNA polymerase complexes, composed of viral phospho- (P) protein and large (L) protein subunits. Transcription follows the "stop-start" model first described for vesicular stomatitis virus (VSV), the result of which is a gradient of transcription in which genes near the 3′ end of the genome are transcribed more abundantly than genes near the 5′ end (reviewed in Whelan et al., 2004). Later in the infectious cycle, the viral polymerase enters a replication mode in which viral genomes are not transcribed, but rather are replicated in a two-step process that involves first the production of positive-sense antigenomes from genomic templates, and subsequently the production of negative-sense genomes from antigenomic templates (reviewed in Lamb and Parks, 2006). Newly-synthesized viral proteins and RNPs assemble together at infected cell plasma membranes in preparation for particle budding, which completes the cycle. Additional functions, including the disabling of host innate antiviral responses, are carried out by viral V proteins and other accessory proteins (reviewed in Horvath, 2004).

Here, recent progress in understanding paramyxovirus particle formation is reviewed. Contributions of various viral components, as well as host proteins, to virus assembly and release are discussed, as well as parameters that lead to successful trafficking of viral components to assembly sites from which virions bud.

2. Central role of M proteins in paramyxovirus particle formation

Infectious paramyxovirus particles can be formed only after all the structural components of the viruses, including viral glycoproteins and viral RNPs, have assembled together at selected sites on infected cell plasma membranes. Viral M proteins are the organizers of this assembly process. These highly abundant viral proteins bind directly to cellular membranes and occupy a central position that allows interaction both with viral RNP cores and also with viral glycoproteins via the cytoplasmic tails (Fig. 1A). Thus, M proteins are adapters that link together the structural components of virions, driving their assembly. Although structural studies of paramyxovirus M proteins have generally proved difficult, as these relatively hydrophobic proteins are prone to self-aggregation, these difficulties were recently overcome in the case of the HRSV M protein, allowing a determination of its atomic structure (Money et al., 2009). The HRSV M protein is composed of two domains, each consisting mainly of beta sheets. The domains are separated by a short, unstructured linker region. The overall structure is quite similar to that of Ebola virus matrix protein (Fig. 4A–B); (Dessen et al., 2000;Money et al., 2009). An extensive positively-charged surface extends across both domains as well as the linker region, and this likely directs electrostatic interactions between the M protein and the phospholipid head groups of membranes (Fig. 4C). This finding is generally consistent with previous biochemical studies, which implicated a combination of electrostatic and hydrophobic contributions to membrane binding by paramyxovirus M proteins (Caldwell and Lyles, 1986;Riedl et al., 2002;Stricker et al., 1994;Subhashri and Shaila, 2007), similar to membrane binding by Ebola virus VP40 (Jasenosky et al., 2001;Ruigrok et al., 2000) and by VSV M protein (Chong and Rose, 1993;Ye et al., 1994).

Direct evidence for the critical role of M proteins in paramyxovirus assembly has been obtained through study of viruses harboring M protein mutations. Early studies relied on Sendai virus temperature-sensitive mutants in which M protein failed to accumulate to

threshold levels at nonpermissive temperatures. This resulted in failure to produce virus particles (Kondo et al., 1993; Yoshida et al., 1979). Additional correlations between M protein defects and virus assembly impairments were made with altered measles virus isolates derived from patients with subacute sclerosing panencephalitis (SSPE). SSPE is a rare condition that results from persistent measles virus infection, causing fatal neurological disease years after initial acute infection with measles virus. SSPE strains of measles virus frequently encode hypermutated and unstable M proteins and are also found to exhibit severe defects in particle assembly (reviewed in Rima and Duprex, 2005). Unequivocal confirmation of the key roles played by paramyxovirus M proteins in particle assembly came with the advent of reverse genetics technology in the 1990s, allowing generation of negative-sense RNA viruses entirely from cloned cDNA (Conzelmann, 2004). Recombinant measles and recombinant Sendai viruses that completely lack M genes have been generated and both of these viruses exhibit near-complete defects in particle formation (Cathomen et al., 1998a; Inoue et al., 2003). Particle production defects have also been observed in recombinant measles virus in which wildtype M protein is replaced with hypermutated M protein derived from an SSPE strain (Patterson et al., 2001), recombinant measles virus in which a highly-conserved valine residue at position 101 has been changed to alanine, resulting in poor M protein stability (Runkler et al., 2007), and recombinant Sendai virus in which M protein accumulation can be prevented through the use of siRNA (Mottet-Osman et al., 2007). Taken together, these studies demonstrate convincingly that efficient paramyxovirus particle formation can occur only in the presence of a threshold level of functioning M protein.

For many paramyxoviruses, M proteins expressed in the absence of other viral proteins are sufficient for the release of virus-like particles (VLPs) from transfected cells. M proteins of hPIV1 (Coronel et al., 1999), Sendai virus (Sugahara et al., 2004; Takimoto et al., 2001), NDV (Pantua et al., 2006), Nipah virus (Ciancanelli and Basler, 2006; Patch et al., 2007), and measles virus (Pohl et al., 2007; Runkler et al., 2007) are all able to elicit efficient particle budding from transfected cells when expressed alone. For NDV, membrane deformation and vesicle budding have even been reconstituted in vitro using purified M protein and unilamellar vesicles (Shnyrova et al., 2007), demonstrating that all of the activities necessary for inducing curvature and fission of a membrane are contained within M protein. In some cases, M protein-directed VLP production from transfected cells becomes even more efficient when the M proteins are co-expressed with other viral components, such as glycoproteins, nucleocapsid proteins, and C proteins (Table 1; and discussed below). Some paramyxovirus M proteins lack the ability to direct efficient VLP production when expressed alone in cells. PIV5 and rinderpest virus M proteins expressed by themselves in transfected cells do not elicit detectable VLP production (Schmitt et al., 2002; Subhashri and Shaila, 2007), and in this respect are similar to the influenza A virus matrix protein (Chen et al., 2007). Mumps virus M protein expression by itself leads to particle release that is barely detectable (Li et al., 2009). For PIV5 and mumps virus, VLP production becomes highly efficient when the M proteins are expressed together with other viral proteins (Table 1). Hence, the requirements for efficient VLP production differ among paramyxoviruses. However, M protein appears generally to be the major requirement for paramyxovirus VLP production, even in the cases where its expression is insufficient, as VLPs cannot be formed in its absence (Li et al., 2009; Schmitt et al., 2002; Teng and Collins, 1998), whereas for influenza A virus the major requirement for VLP budding is the hemagglutinin (HA) glycoprotein (Chen et al., 2007).

3. Cooperation among viral proteins during particle production

3.1 Viral glycoproteins

Paramyxovirus particles are covered with spike layers consisting of the viral attachment and fusion glycoproteins (Fig. 2). The viral glycoproteins assemble together with M proteins in infected cells, clustering on plasma membranes at locations from which virus particles will bud (Fig. 1). The cytoplasmic tails of paramyxovirus glycoproteins interact with the M proteins to organize virus assembly. Evidence for these interactions has been obtained through a variety of experimental approaches. Fluorescence microscopy experiments have documented co-localization of paramyxovirus M proteins and glycoproteins in virus-infected cells. In the case of PIV5, M proteins and glycoproteins co-localize in clusters on infected cell plasma membranes. However, this clustering is lost in cells infected with mutant viruses in which the cytoplasmic tail of HN protein, or the cytoplasmic tails of both HN and F proteins, have been truncated (Schmitt et al., 1999;Schmitt et al., 2005;Waning et al., 2002). In cells infected with the Edmonston vaccine strain of measles virus, M protein co-localizes with the viral F and H glycoproteins, but no co-localization could be observed between M protein and cytoplasmic tail-deleted viral glycoproteins (Moll et al., 2002). Co-localization studies have also been performed in cells transfected to produce the HRSV M and G proteins. Co-localization between the wildtype proteins was observed, but co-localization was lost upon removal of the first six amino acid residues of the G protein cytoplasmic tail (Ghildyal et al., 2005b). Co-immunoprecipitation studies using purified VLPs have been conducted to explore interactions involving the glycoproteins of NDV. Specific interactions between the NDV HN and M proteins, and also between the F and NP proteins, were detected. No evidence for direct interaction between the F and M proteins was obtained, however (Pantua et al., 2006). Further experimental support for specific interactions between viral glycoproteins and M proteins has been obtained by measuring the association of M proteins with detergent-resistant raft membranes (discussed further in section 5.2). Sendai virus M protein binds to cellular membranes intrinsically, but it does not associate strongly with detergent-resistant raft membranes when expressed by itself. The Sendai virus HN and F glycoproteins, however, are intrinsically sorted to raft membranes. When the viral glycoproteins are co-expressed together with M protein, M protein becomes raft membrane-associated (Ali and Nayak, 2000). Similar results were obtained with HRSV M protein, which also binds membranes intrinsically, but does not associate with detergent-resistant raft membranes unless the HRSV F protein is co-expressed, suggesting that the F protein pulls M protein into detergent-resistant membrane microdomains (Henderson, 2002). Together, these observations support a model in which viral glycoproteins and viral M proteins assemble together at specific locations on cellular membranes through interactions involving the glycoprotein cytoplasmic tails.

Proper assembly of paramyxovirus glycoproteins together with M proteins in infected cells is necessary for selective incorporation of the glycoproteins into budding particles, and in many instances is also necessary for the budding process itself to become efficient. In the case of PIV5, the F and HN glycoproteins have redundant functions important for efficient virus budding. Recombinant virus in which the HN protein cytoplasmic tail is truncated buds particles poorly, and this defect is more pronounced in double mutant virus in which both HN and F protein cytoplasmic tails have simultaneously been truncated (Schmitt et al., 1999; Waning et al., 2002). These findings parallel earlier results obtained with influenza A virus, in which simultaneous truncation of the HA and neuraminidase (NA) protein cytoplasmic tails resulted in severe particle production and particle morphology defects, while single cytoplasmic tail truncations led to only minor defects (Jin et al., 1997). For Sendai virus, the HN glycoprotein appears to be dispensable for the production of virus particles (Markwell et al., 1985; Portner et al., 1974; Stricker and Roux, 1991), although particle morphology may be altered in the absence of HN protein (Hirayama et al., 2006).

Truncation of the Sendai virus F protein cytoplasmic tail led to inefficient virus release (Fouillot-Coriou and Roux, 2000). Some alterations to the HN protein led to deficient incorporation of the protein into virions, with no detrimental effects on virus budding, while severe truncation of the HN protein cytoplasmic tail caused poor virus release (Fouillot-Coriou and Roux, 2000). The importance of glycoproteins for measles virus particle formation has been inferred from observations with assembly-defective SSPE strains, which frequently harbor F proteins with mutated cytoplasmic tails (Cattaneo et al., 1988; Schmid et al., 1992). Alterations to the F and H protein cytoplasmic tails in recombinant viruses led to reduced incorporation of the viral glycoproteins into virions, along with increased nonspecific incorporation of cellular proteins (Cathomen et al., 1998b). These alterations also led to rapid and extensive syncytia formation within infected cell monolayers (Cathomen et al., 1998b). Interestingly, assembly-defective recombinant PIV5 with truncated HN protein cytoplasmic tail also caused rapid and extensive syncytia formation (Schmitt et al., 1999). Vaccine strains of measles virus, including the Edmonston strain, harbor adaptations in their M proteins that confer enhanced virus assembly and release compared with wildtype measles virus isolates (Tahara et al., 2007). These adaptations enhance interaction with the H protein cytoplasmic tail judged by co-localization studies, and reduce syncytia formation (Tahara et al., 2007). Together, these findings suggest that assembly of viral M proteins together with viral glycoproteins may benefit paramyxovirus particle formation and release, but at the same time may impair F protein-mediated cell-cell fusion. This may allow measles virus to optimize its mode of spread (cell-cell fusion versus budding) by acquiring mutations in the M protein, thereby modulating the interaction with the H protein cytoplasmic tail (Tahara et al., 2007).

The roles of viral glycoproteins in paramyxovirus assembly have also been examined in the context of VLPs produced from transfected cells. Although M proteins are sufficient for VLP production in many cases, expression of glycoproteins together with M proteins generally leads to production of VLPs that incorporate the viral glycoproteins and resemble paramyxovirus virions morphologically (Ciancanelli and Basler, 2006; Pantua et al., 2006; Patch et al., 2007). Some paramyxovirus glycoproteins have intrinsic exocytosis activities and are released as particles even when expressed alone. These include the Sendai virus F protein (Sugahara et al., 2004; Takimoto et al., 2001), the measles virus F protein (Pohl et al., 2007), and the Nipah virus F and G proteins (Ciancanelli and Basler, 2006; Patch et al., 2007). Other paramyxovirus glycoproteins lack inherent exocytosis activities, including the Sendai virus HN protein (Sugahara et al., 2004; Takimoto et al., 2001), and the glycoproteins of PIV5 (Schmitt et al., 2002), NDV (Pantua et al., 2006), and mumps virus (Li et al., 2009). In some cases, viral glycoproteins and M proteins cooperate with one another, resulting in VLP release that is more efficient than that obtained when either protein is expressed alone. For example, production of Sendai VLPs, driven by M protein, is made more efficient upon co-expression of the viral F protein (Sugahara et al., 2004; Takimoto et al., 2001). The Sendai virus HN protein, on the other hand, lacks the ability to enhance VLP production (Sugahara et al., 2004; Takimoto et al., 2001). Viral glycoprotein expression is required for efficient production of mumps VLPs and PIV5-like particles. Mumps VLP production is most efficient upon co-expression of the viral M, NP, and F proteins (Li et al., 2009). Co-expression of the mumps virus M, NP, and HN proteins leads to VLP production that is considerably less efficient. Hence, similar to the Sendai virus glycoproteins, the mumps virus glycoproteins are not equal contributors to virus assembly, with F protein playing the more important role. The PIV5 glycoproteins, in contrast, appear to have more equal, and seemingly redundant, functions during VLP production. Here, similar and highly efficient VLP production can be achieved either through co-expression of the M, NP, and F proteins, the M, NP, and HN proteins, or the M, NP, F, and HN proteins (Schmitt et al., 2002). Neither of the PIV5 glycoproteins can function for VLP production when their cytoplasmic tails have been truncated, in agreement with results obtained using recombinant

viruses (Schmitt et al., 1999; Waning et al., 2002). These results suggest that some paramyxovirus glycoproteins, when present at virus assembly sites, can act to increase the efficiency of particle production. However, other viral glycoproteins, including those of NDV, Nipah virus, and measles virus, do not enhance the efficiency of M protein-driven VLP release from transfected cells (Ciancanelli and Basler, 2006; Pantua et al., 2006; Patch et al., 2007; Pohl et al., 2007). Hence, while assembly of paramyxovirus glycoproteins together with viral M proteins may have universal importance for efficient incorporation of the glycoproteins into budding particles, this assembly affects the efficiency of particle release for only a subset of paramyxoviruses (Table 1).

3.2 Viral nucleocapsid proteins

Paramyxovirus nucleocapsid proteins bind and encapsidate genomic RNA, forming helical RNP complexes in which the RNA is tightly packaged and resistant to exogenously added RNase. The structure of HRSV N protein bound to RNA was recently determined by x-ray diffraction and cryoelectron microscopy (Tawar et al., 2009). RNA is wrapped around a decameric ring of N protein, fitting along a basic surface groove. This arrangement is consistent with a model in which the viral RNA-dependent RNA polymerase is able to recognize its template even without disassembly of the RNP. From the analogy of tobacco mosaic virus, it is assumed that RNA encapsidation starts from a nucleocapsid protein-binding nucleation site. In virus-infected cells, nascent genomic RNA is efficiently encapsidated coupled with RNA synthesis, while viral mRNAs are not encapsidated. It is therefore assumed that the promoter sequences (the leader and trailer sequences) function as nucleation sites (Blumberg et al., 1983; Lamb and Parks, 2006). On the other hand, it is known that paramyxovirus nucleocapsid proteins expressed in mammalian cells bind intracellular host RNAs non-specifically and form nucleocapsid-like structures (Buchholz et al., 1993; Coronel et al., 1999; Errington and Emmerson, 1997; Juozapaitis et al., 2005; Schmitt et al., 2002; Spehner et al., 1991; Sugahara et al., 2004), indicating that RNA encapsidation may occur in some circumstances even without the aid of a nucleation site.

Once formed, RNPs must be incorporated into budding virus particles. Although paramyxovirus genomes are nonsegmented, it is not necessarily the case that all budding particles receive only a single copy of genomic RNA. Some virions are released that contain multiple genome copies (Loney et al., 2009; Rager et al., 2002). Selective incorporation of homologous genomes into virions has been demonstrated in experiments in which cells were transfected to produce the hPIV1 NP protein and also were infected with Sendai virus. Nucleocapsid structures containing hPIV1 NP protein were produced in the cells, but these were largely excluded from Sendai virions (Coronel et al., 2001). Selectivity of genome incorporation by genome length has also been reported; short defective-interfering genomes of Sendai virus were incorporated into budding particles much less efficiently than full-length genomes (Mottet and Roux, 1989). Selective genome incorporation based on the polarity of the viral RNA also occurs, with (−)-sense genomes incorporated more efficiently into budding particles than (+)-sense antigenomes. However, for paramyxoviruses such as Sendai virus, this packaging bias in favor of genomes is very weak, compared to the more stringent preferential packaging of (−)-sense genomes into rhabdovirus virions (Kolakofsky and Bruschi, 1975; Mottet and Roux, 1989). The Sendai virus C protein is thought to regulate viral RNA synthesis from the promoters, and excessive amounts of (+)-sense antigenomes are synthesized in partial C-knockout virus-infected cells. Virions released from the infected cells contain (−) and (+)-sense RNA genomes in a ratio similar to that present inside the infected cells, resulting in an overabundance of noninfectious virions containing (+)-sense antigenomic viral RNA (Irie et al., 2008a).

Incorporation of genomes into budding virions is likely driven by interactions between viral matrix proteins and nucleocapsids at virus assembly sites. Biochemical evidence supports a

direct interaction between paramyxovirus matrix and nucleocapsid proteins (Iwasaki et al., 2009; Markwell and Fox, 1980; Yoshida et al., 1976). For measles virus, yeast two-hybrid binding assays have mapped this interaction to the C-terminal portion of the N protein (Iwasaki et al., 2009). In transfected cells, expression of measles virus M protein shifts the localization of wt N protein (but not N protein harboring a C-terminal mutation) so that a portion is plasma membrane associated and co-localized with M protein (Iwasaki et al., 2009). Similarly, RNPs are observed at the plasma membrane in cells infected with standard measles virus, but not in cells infected with recombinant measles virus harboring an unstable M protein (Runkler et al., 2007). Together, these studies support a model in which M protein is responsible for targeting RNPs to virus assembly sites on plasma membranes.

Incorporation of nucleocapsid-like structures into budding VLPs has been documented in transfected cells. For example, co-expression of the hPIV1 M and NP proteins in cells led to production of VLPs that enclose nucleocapsid-like structures (Coronel et al., 1999). Similar results were obtained with Sendai VLPs (Sugahara et al., 2004) and Nipah VLPs (Patch et al., 2007). Nucleocapsid-like structures can also be incorporated into NDV-like particles, however in this case nucleocapsid incorporation was poor unless viral glycoproteins were expressed in addition to the M and NP proteins (Pantua et al., 2006). For PIV5 and mumps virus, NP proteins not only incorporate into budding VLPs, they actually increase the efficiency of VLP production (Li et al., 2009; Schmitt et al., 2002). It is possible that for these viruses a requirement for NP protein during assembly could be beneficial through minimizing the release of noninfectious particles that lack viral genomes.

3.3 Viral C proteins

Some paramyxoviruses express accessory C proteins from their P genes via overlapping reading frames. For example, Sendai virus encodes a nested set of four C proteins from its P gene through use of alternative start codons that are recognized by translation machinery either by leaky scanning in the case of C and C′, or by a type of ribosomal shunting in the case of Y1 and Y2 (Lamb and Parks, 2006). C proteins appear to be multifunctional, with activities that regulate viral RNA synthesis (Cadd et al., 1996; Curran et al., 1992) and that counteract innate immune responses of host cells (Garcin et al., 1999; Gotoh et al., 1999). The longer C proteins (C and C′) contain N-terminal membrane-targeting sequences, while the shorter C proteins (Y1 and Y2) lack these sequences (Marq et al., 2007). A role for C proteins in Sendai virus budding was first suggested based on experiments with 4C(−) virus, which lacks the ability to produce any of the four C proteins (Kurotani et al., 1998). This virus replicates to titers that are about two logs less than wildtype Sendai virus and fails to assemble and release virus particles effectively, despite efficient synthesis of viral mRNAs, proteins, and genomic RNA (Hasan et al., 2000). Defective assembly of 4C(−) virus was observed in multiple cell types, including Vero cells that fail to produce IFN-β, suggesting a virus assembly function of C protein that is separate from its function in counteracting innate immune defenses. Separation of C protein assembly and immune-evasion functions is also supported by results obtained with altered Sendai virus Cm*, which harbors a C protein point mutation and fails to block the Jak/Stat pathway, yet exhibits no virus assembly defect (Kato et al., 2007). Although the above findings support a role for C protein in Sendai virus assembly, such a role could not be confirmed by Gosselin-Grenet et al., who used a somewhat different experimental system. Here, a 4C knockout virus was employed in which C protein fused to GFP was re-introduced as a separate transcriptional unit in the virus genome. GFP/C protein expression was ablated using siRNA, with no apparent effect on virus particle production (Gosselin-Grenet et al., 2007). This approach has an advantage in that it avoids passaging of a seriously debilitated virus, in which selective pressure could potentially cause unintended variants of the virus to emerge. The approach was also limited, however, in that siRNA depletion was incomplete (about 30% of normal GFP/C protein

levels remained), raising the possibility that C protein could initially have been present in excess of what is needed for virus assembly. Further investigations on the importance of C proteins in Sendai virus assembly have been carried out in the context of VLPs produced from transfected cells. Expression of Sendai virus C protein together with M protein results in VLP production that is more efficient (by 2–3 fold) than that obtained when M protein is expressed alone (Sugahara et al., 2004). C and C′ proteins are each capable of enhancing Sendai VLP production, while the Y1 and Y2 proteins that lack membrane targeting sequences are unable to enhance VLP production (Irie et al., 2008b; Sakaguchi et al., 2005). A model has been proposed in which C proteins function for virus budding by interacting with the host Class E protein Aip1/Alix, recruiting it to Sendai virus assembly sites on plasma membranes (Irie et al., 2008b; Sakaguchi et al., 2005). Evidence in support of such a role for Aip1/Alix, and potential roles for other host proteins in paramyxovirus budding, are discussed below.

4. Role of host proteins in paramyxovirus budding

4.1 Class E proteins and ubiquitin

Enveloped viruses typically do not encode all of the machinery that is necessary to bud particles. Instead, host machinery is manipulated to allow efficient virus exit. Retroviruses in many cases employ protein-protein interaction sequences (late domains) within their Gag polypeptides to recruit host factors to virus assembly sites (reviewed in Bieniasz, 2006; Calistri et al., 2009). Absence of these sequences in some instances leads to defects in the very late stages of virus release, hence the term late domain. Late budding defects are characterized by accumulation of budding structures tethered to cellular membranes that have morphology consistent with virions, but cannot be released by membrane fission (reviewed in Demirov and Freed, 2004). Several distinct types of viral late domain sequences have been characterized from retroviral Gag proteins, including P(T/S)AP, PPxY, and $YP(x)_nL$. Matrix proteins of some negative-strand RNA viruses such as VSV (Craven et al., 1999; Harty et al., 1999; Jayakar et al., 2000), rabies virus (Wirblich et al., 2008), Ebola virus (Harty et al., 2000; Licata et al., 2003; Martin-Serrano et al., 2001), LCMV (Perez et al., 2003), and Lassa fever virus (Perez et al., 2003) contain these same late domain sequences, suggesting that the overall strategy of recruiting host machinery via late domains is conserved even among distantly-related viruses. Paramyxoviruses, however, generally lack these well-characterized late domain sequences within their matrix proteins, indicating that the logistics of host factor recruitment must be different for these viruses.

Cellular factors recruited by late domains in many cases are Class E proteins that are part of the vacuolar protein sorting (Vps) pathway of the cell. These proteins form endosomal sorting complexes required for transport (ESCRTs) that allow for multivesicular body formation and the downregulation of cellular membrane proteins (reviewed in Stuffers et al., 2009). ESCRT complexes are dismantled and recycled with the aid of Vps4 AAA-ATPases, and disruption of the Vps sorting pathway through expression of dominant-negative (DN) Vps4 protein mutants blocks the budding of many retroviruses (reviewed in Bieniasz, 2006). Similar findings have been obtained with some paramyxoviruses. PIV5 budding (Schmitt et al., 2002) and mumps VLP budding (Li et al., 2009) were both inhibited upon expression of DN Vps4 proteins, suggesting that these viruses rely on ESCRT machinery during virus exit, even though they lack classical late domains that would mediate ESCRT recruitment. DN Vps4 protein expression also inhibits the budding of other negative-strand RNA viruses including Ebola virus (Licata et al., 2003), and Lassa fever virus (Urata et al., 2006), although in these cases classical late domain sequences are contained within the viral matrix proteins. Although it is clear that a wide range of enveloped viruses employ host ESCRT machinery during budding, it has recently become apparent that some enveloped viruses do not. Among paramyxoviruses, budding of HRSV (Utley et al., 2008) and budding of Nipah

VLPs (Patch et al., 2008) occur efficiently even in the presence of DN Vps4 protein expression. These results parallel earlier observations of ESCRT-independent budding with VSV (Irie et al., 2004) and influenza virus (Chen et al., 2007), raising the possibility that some viruses employ host pathways during budding that are ESCRT-independent, or perhaps have the ability to bud even without the aid of host machinery (reviewed in Chen and Lamb, 2008).

Potential links between Sendai virus budding and host ESCRT machinery have been examined in some detail. The Sendai virus C protein, a nonstructural accessory protein of the virus, binds to Aip1/Alix, the same protein that is recruited by retroviral $YP(x)_nL$ late domains. However, Sendai virus C protein lacks $YP(x)_nL$ and instead binds to Aip1/Alix via a domain within its C-terminal region (Sakaguchi et al., 2005). Expression of C protein together with Aip1/Alix in transfected cells re-localized a portion of Aip1/Alix to the plasma membrane together with C protein (Irie et al., 2008b). Sendai VLP production, driven by M protein, was enhanced when M protein was expressed together with C protein (Sugahara et al., 2004). Mutant C proteins that fail to interact with Aip1/Alix (Sakaguchi et al., 2005) or that fail to bind membranes (Irie et al., 2008b) could not enhance VLP production. Independently of its interaction with C protein, Aip1/Alix has been shown to interact with the Sendai virus M protein (Irie et al., 2007). This interaction is mediated by a YLDL motif within M protein. Mutations in the YLDL motif or depletion of Aip1/Alix with siRNA inhibited the production of Sendai VLPs (Irie et al., 2007). These observations support a model in which Aip1/Alix can be recruited to sites of Sendai virus assembly on plasma membranes either through C protein interaction or through M protein interaction, resulting in enhanced VLP production. It is unclear at present why multiple mechanisms for Aip1/Alix recruitment seem to be necessary for optimal production of Sendai VLPs, though a similar potential redundancy has been reported for hPIV2, in which the V and NP proteins were found to bind Aip1/Alix independently (Nishio et al., 2007). Questions also remain concerning the importance of Aip1/Alix recruitment in the course of an actual Sendai virus infection, as conflicting results have been obtained when examining the effect of Aip1/Alix depletion on Sendai virus budding (Gosselin-Grenet et al., 2007; Sakaguchi et al., 2005). These reports also conflict regarding the effect of DN Vps4 protein expression on Sendai virus budding. Sakaguchi et al. (2005) found that transfection of cells with DN Vps4 protein reduced budding of virions from cells transfected with infectious Sendai virus nucleocapsids, while Gosselin-Grenet et al. (2007) observed no inhibition in the release of virions from cells transfected to express DN Vps4 protein and infected with Sendai virus, even though PIV5 virion release was substantially inhibited in parallel experiments. When examining VLP production, DN Vps4 protein expression was found to only affect the accelerated release of VLPs that occurs in the presence of the viral C protein, and not M-alone VLP production (Irie et al., 2008b), consistent with the possibility that C protein enhances VLP budding through recruitment of ESCRT machinery.

An interaction between the PIV5 M protein and host protein angiomotin-like 1 (AmotL1), was characterized recently using yeast two-hybrid as well as co-immunoprecipitation assays (Pei et al., 2009). AmotL1 harbors two PPxY motifs, and recruitment of AmotL1 to virus assembly sites could in theory allow indirect recruitment of the same WW domain-containing host proteins that are directly recruited by viruses that employ PPxY late domains. PIV5 budding was inhibited by siRNA-mediated depletion of AmotL1 from cells, and overexpression of M-binding AmotL1-derived polypeptides blocked the production of PIV5-like particles (Pei et al., 2009). AmotL1 localizes to tight junctions and apical membranes of epithelial cells (Sugihara-Mizuno et al., 2007), and it has been suggested that this protein or other motin proteins could be involved in assembly of PIV5 on apical membranes (Pei et al., 2009).

Ubiquitin is thought to play a role in the budding of some enveloped viruses, likely stemming from its relationship with ESCRT machinery (reviewed in Martin-Serrano, 2007; Morita and Sundquist, 2004). Monoubiquitination or multiple monoubiquitination of cellular membrane proteins is a sorting signal for endocytosis and likely allows recognition by ESCRT proteins, several of which harbor well-characterized ubiquitin-binding domains (Hicke and Dunn, 2003; Shields et al., 2009). Several lines of evidence support a role for ubiquitin in the budding of certain retroviruses, including HIV-1. For example, ubiquitin has been detected in retrovirus particles (Ott et al., 2000; Putterman et al., 1990), multiple monoubiquitination of retroviral Gag proteins has been observed (Gottwein and Krausslich, 2005), and budding of some retroviruses is inhibited upon treatment of cells with proteasome inhibitors, which deplete cellular pools of free ubiquitin (Patnaik et al., 2000; Schubert et al., 2000). Ebola VP40 and VSV M protein can act as substrates for ubiquitin attachment in vitro, providing a link between ubiquitin and negative-strand RNA virus budding (Harty et al., 2000; Harty et al., 2001). In addition, VLP production driven by VSV M protein is proteasome inhibitor sensitive (Harty et al., 2001). Nedd4 mediated ubiquitination of Ebola VP40, detected in cells transfected to express VP40 and hemagglutinin-tagged ubiquitin (HA-Ub), is inhibited by expression of ISG15, a ubiquitin-like protein whose expression is induced by interferon (Malakhova and Zhang, 2008; Okumura et al., 2008). These findings support the ideas that enveloped virus budding may involve the ubiquitin-proteasome pathway, and that negative-strand RNA virus matrix proteins may be substrates for ubiquitin attachment.

Some evidence in support of ubiquitin involvement in paramyxovirus budding has been obtained. Release of PIV5 virions and VLPs from 293T cells is significantly impaired upon proteasome inhibitor treatments, suggesting that the ubiquitin-proteasome system may play an important role in some aspect of PIV5 assembly and/or budding (Schmitt et al., 2005). Potential ubiquitination of measles virus M protein has been observed in cells transfected to express measles virus M protein together with HA-Ub (Pohl et al., 2007). On the other hand, proteasome inhibitor treatments failed to inhibit the budding of HRSV from Hep-2 cells or the budding of Nipah VLPs from 293T cells, suggesting that differences may exist among the paramyxoviruses in the degree of dependence on ubiquitin for budding (Patch et al., 2008; Utley et al., 2008). For Sendai virus, sensitivity to proteasome inhibitor treatment was found to be cell type-dependent. Budding from LLC-MK2 cells was highly sensitive to proteasome inhibitor treatment, budding from CV-1 cells was less sensitive, and budding from A549 cells was proteasome inhibitor resistant (Watanabe et al., 2005). This cell type-dependent behavior underscores a more general complexity inherent in the study of host factors important for virus replication, in that the relative abundances of these factors are likely to differ among cell types, perhaps reinforcing the importance of studying these virus-host interactions, where possible, in cell types which most resemble those found in the natural hosts of infection. Overall, the observations described here are consistent with the involvement of ubiquitin in the assembly and release of a subset of paramyxoviruses, though an understanding of the specific role of ubiquitin will necessitate further examination.

4.2 M protein sequences that may function to allow host factor recruitment

Although paramyxovirus M proteins generally lack classical late domain sequences, a number of alternative sequences have been identified within M proteins that are important for budding activity and that may function to recruit host factors, similar to retroviral late domains. The sequence 20-FPIV-23 within PIV5 M protein was identified based on its ability to restore VLP budding function to PTAP-disrupted HIV-1 Gag protein (Schmitt et al., 2005). Mutation of either the phenylalanine or proline residues within this sequence drastically impaired the budding function of PIV5 M protein (Schmitt et al., 2005). Mumps virus M protein contains a similar sequence, 21-FPVI-24, and mumps VLP production was

inhibited upon mutation of the proline residue within this sequence (Li et al., 2009). FPIV and FPIV-like sequences are presumed to function by mediating host factor recruitment to virus assembly sites, but the binding partner(s) for these sequences have not yet been identified.

Two sequences in NiV M protein that are important for VLP production have been identified: 62-YMYL-65 and 92-YPLGVG-97 (Ciancanelli and Basler, 2006; Patch et al., 2008). The YMYL sequence was able to functionally replace the PTAPPEY late domain of Ebola virus VP40, as appending this sequence to late domain-defective VP40 restored VLP budding function (Ciancanelli and Basler, 2006). Mutation of YMYL inhibited the VLP budding function of NiV M protein, and led to a re-localization of the protein to the cell nucleus (Ciancanelli and Basler, 2006). The sequence YPLGVG within NiV M protein contains residues that are well conserved among paramyxoviral M proteins (Patch et al., 2008). Mutation of this sequence disrupted M protein-driven VLP production, and deletion of the sequence led to nuclear localization of NiV M protein. YPLGVG was unable to rescue VLP budding function of late domain-defective Ebola virus VP40; however, the appearance of filamentous projections similar to those observed in cells expressing wt VP40 or wt NiV M protein was restored (Patch et al., 2008). Cellular binding partners for YMYL and YPLGVG sequences have not yet been identified. Interestingly, sequences resembling YPLGVG are found in the Ebola virus and Marburg virus VP40 proteins, and mutation of these sequences disrupted VP40 stability, intracellular localization, and VLP production (Liu et al., 2009). Thus, YPLGVG-like sequences appear to be important for the functions of divergent negative-strand RNA virus matrix proteins.

Sendai virus M protein possesses a sequence, 49-YLDL-52, which binds Alix/Aip1. Mutation of YLDL caused a reduction in VLP release that could not be rescued by the inclusion of other Sendai virus proteins (Irie et al., 2007). The YLDL sequence could not be replaced by P(T/S)AP, PPxY, or $YP(x)_nL$ late domains for the budding of Sendai VLPs. YLDL-Aip1/Alix interaction appears to be distinct from $YP(x)_nL$-Aip1/Alix interaction, as the Sendai virus YLDL sequence interacts with Aip1/Alix residues 1–211 while $YP(x)_nL$ interacts with residues 409–715 (Irie et al., 2007).

5. Virus assembly sites

5.1 Paramyxovirus assembly on apical membranes of polarized cells

Polarized epithelial cells that line body surfaces possess apical and basolateral sides, with the apical sides facing outward and the basolateral sides facing inward towards the underlying tissue. Several paramyxoviruses target epithelial cells of the respiratory tract for infection and assemble and bud from the apical surfaces of these cells. HRSV, Sendai virus, PIV5, hPIV3, and measles virus all bud preferentially from the apical surfaces of polarized cells (Blau and Compans, 1995; Bose et al., 2001; Roberts et al., 1995; Rodriguez-Boulan and Sabatani, 1978). Other respiratory viruses, including influenza A virus, also bud apically (Rodriguez-Boulan and Sabatani, 1978), while VSV and Marburg virus bud basolaterally (Sänger et al., 2001). The direction of virus budding from polarized cells can influence whether a paramyxoviral infection remains localized to the respiratory epithelia, facilitating transmission between hosts, or disseminates systemically, which in some cases favors establishment of persistent infections (reviewed in Schmitt and Lamb, 2004; Takimoto and Portner, 2004).

In many cases, viral glycoproteins have intrinsic sorting signals that target them to apical or basolateral cell surfaces. However, it does not appear that viral glycoproteins generally control the polarity of paramyxovirus budding. Instead, budding polarity appears to be determined mainly by the viral M proteins. This is likely the case for HRSV, in which the F

glycoprotein is intrinsically targeted to the apical surface (Batonick et al., 2008; Brock et al., 2005), yet recombinant HRSV that lacks the F gene still buds apically (Batonick et al., 2008). In fact, even HRSV lacking all three glycoproteins (F, G, and SH) buds apically, implying that internal viral proteins control the direction of budding (Batonick et al., 2008). An additional example is provided by measles virus, in which the viral glycoproteins intrinsically target to basolateral cell surfaces when expressed alone. In virus-infected cells, a substantial fraction of the measles virus F and H glycoproteins is re-routed to apical cell surfaces from which virus particles bud (Maisner et al., 1998). In cells infected with recombinant measles virus lacking an M gene, the measles virus glycoproteins fail to re-route to the apical side, indicating that the direction of measles virus budding, as well as the redirection of measles virus glycoproteins, is likely controlled by M protein (Naim et al., 2000). Intrinsic targeting of measles virus glycoproteins to basolateral membranes may be important for pathogenesis, as it favors cell-to-cell transfer of infection and may contribute to systemic spread in vivo (Moll et al., 2002). M protein is also thought to define the apical budding of Sendai virus. A pantropic mutant Sendai virus, F1-R, buds both apically and basolaterally and causes systemic infection in mice, whereas wt Sendai virus budding is restricted to apical surfaces, resulting in localized infection of the respiratory tract (Tashiro et al., 1996; Tashiro et al., 1992). The altered budding polarity is attributed to mutations in the M protein, as the mutant M protein disrupts the microtubule network of the cell and alters maintenance of cell polarity. Whereas wt Sendai virus M protein redirects the bipolar transport of mutant F protein from the F1-R virus to the apical cell surface, F1-R M protein does not (Tashiro et al., 1996). These findings all support a key role for paramyxovirus M proteins in the polarized assembly and release of virus particles.

Apical transport and recycling of host proteins in polarized cells is facilitated by the apical recycling endosome (ARE), which is enriched in Rab11a. Recent studies have demonstrated that efficient polarized budding of HRSV depends on proper ARE function (Brock et al., 2005; Utley et al., 2008). Expression of altered myosin Vb protein disrupted HRSV replication (Brock et al., 2005). The altered protein lacks its myosin motor domain, and expression of this protein in polarized cells disrupts basolateral-to-apical transcytosis. Reduction in HRSV yield was mainly caused by failure of virus assembly at the apical surface. HRSV replication could also be inhibited through expression of a DN Rab11-FIP2 protein that lacks its Rab-binding domain (Utley et al., 2008). Rab11 family interacting proteins (Rab11-FIPs) assist in the regulation of ARE-mediated protein sorting. Reduction in HRSV replication in this case was caused by a late domain-like defect, as judged by retention and significant elongation of assembled viral filaments on infected cells (Utley et al., 2008). Hence, FIP2 protein is required at a late stage of HRSV budding from polarized cells, most likely membrane fission. This may represent a novel, alternative pathway for the polarized budding of some viruses, such as HRSV, which exit from cells via a mechanism that seems to be completely independent of ESCRT machinery.

5.2 Budding of virus particles from lipid raft membranes

Microdomains within lipid bilayers, such as the cholesterol- and sphingolipid-rich raft microdomains, have the potential to act as platforms for virus assembly by providing nucleation points to allow concentration of viral proteins prior to budding (reviewed in Brown and London, 2000; Chazal and Gerlier, 2003; Schmitt and Lamb, 2005; Simons and Ikonen, 1997; Simons and Toomre, 2000; Takimoto and Portner, 2004). In paramyxovirus-infected cells, viral proteins in many cases sort to raft membranes selectively, judged by their association with detergent-resistant membrane (DRM) that resists solubilization with cold nonionic detergents such as Triton-X100. In Sendai virus-infected cells, for example, the viral structural proteins F, HN, M, N, and P are all found at least partially associated with DRM (Gosselin-Grenet et al., 2006). In transfected cells, the F and HN glycoproteins

are associated with DRM when expressed alone or in combination (Sanderson et al., 1995), but the M protein is associated with DRM only when it is co-expressed with F or HN protein, suggesting that interaction between M protein and viral glycoprotein cytoplasmic tails is essential for proper targeting of M protein to raft membrane (Ali and Nayak, 2000). Measles virus structural proteins are also found associated with DRM in virus-infected cells (Manié et al., 2000; Vincent et al., 2000). Here, the F and M proteins are each capable of independently associating with DRM in transfected cells, although M protein DRM association was found to increase upon co-expression of F protein (Pohl et al., 2007). NDV HN, F, and NP proteins all associate with DRM during virus infection, and the DRM-associated viral proteins are ultimately found in released NDV virions (Laliberte et al., 2006). Lipid raft-associated proteins caveolin-1, flotillin-2, and actin are also found in NDV virions (Laliberte et al., 2006). NDV F protein is associated with DRM when expressed alone, and deletions of the F protein cytoplasmic tail inhibit DRM association (Dolganiuc et al., 2003). HRSV F, G, M, and N proteins all associate with DRM in virus-infected cells (Brown et al., 2004; Marty et al., 2004) and several lipid raft markers, including caveolin-1, GM-1, CD-55, and CD58, are found in released HRSV virions or in budding HRSV filaments (Brown et al., 2002; Brown et al., 2004; Jeffree et al., 2003). In transfected cells, HRSV M protein associates with raft membrane only when it is co-expressed with F protein (Henderson, 2002). In cells infected with canine distemper virus (CDV), the H and F glycoproteins are associated with DRM (Imhoff et al., 2007). In sum, the targeting of viral proteins to raft membranes for assembly appears to be a common strategy employed by a variety of different paramyxoviruses.

Evidence that lipid rafts are in fact important during paramyxovirus infections has been obtained mainly through experiments that disrupt raft microdomains by depleting cholesterol with the drug methyl-β-cyclodextrin (MβCD). In Sendai virus infection, MβCD treatment did not impair virion production, however the virions that were released were less infectious than virions released from untreated cells (Gosselin-Grenet et al., 2006). Treatment of CDV-infected cells with MβCD did not affect particle release or infectivity, though treatment of CDV virions with MβCD after they had been released resulted in a loss of infectivity that was restored upon supplementation with cholesterol (Imhoff et al., 2007). NDV particle release was found to be enhanced by MβCD treatment (Laliberte et al., 2006). However, the released particles were somewhat altered in polypeptide composition, displayed abnormal morphology, and were less infectious than particles released from untreated cells (Laliberte et al., 2006). Further characterization of these particles revealed a defect in virus-cell membrane fusion, and a loss of complexes formed between the HN and F glycoproteins in virion envelopes (Laliberte et al., 2007). Infectivity defects have also been observed for NDV virions released from Niemann-Pick syndrome type C cells, a cell type lacking lipid rafts (Laliberte et al., 2007). Overall, these results support a model in which assembly of paramyxovirus components at lipid raft platforms enhances the quality, but not necessarily the quantity, of virus particles that are released.

Recent reports illuminate changes in cholesterol biosynthesis that occur in paramyxovirus-infected cells. 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), an enzyme involved in the formation of endogenous cholesterol, and low-density lipoprotein receptor, involved in cholesterol homeostasis, are upregulated during HRSV infection, judged by microarray analysis (Martinez et al., 2007; Yeo et al., 2009). Levels of HMGCR and squalene monooxygenase, which functions in sterol biosynthesis, are altered during measles virus infection, and depletion of cholesterol from cells through simvastatin-mediated inhibition of HMGCR reduces that amount of infectious measles virus release in an acute infection (Robinzon et al., 2009). Intriguingly, several genes involved in cholesterol biosynthesis are downregulated in cells persistently infected with measles virus compared to those undergoing acute infection (Robinzon et al., 2009), and it has been suggested that the

presence of a cellular mechanism to downregulate cholesterol during virus infection could serve as an innate antiviral mechanism aimed at limiting the budding of infectious particles (Robinzon et al., 2009).

5.3 Virus assembly and the cytoskeleton

Involvement of the actin cytoskeleton in paramyxovirus budding was first proposed based on the detection of large quantities of actin in purified preparations of Sendai virions and measles virions (Lamb et al., 1976; Tyrrell and Norrby, 1978). Other paramyxoviruses incorporate actin into virions as well, including NDV and HRSV (Laliberte et al., 2006; Marty et al., 2004). Sendai VLPs produced upon expression of either M protein or F protein contain actin, and both of these proteins contain sequences important for budding that resemble actin-binding domains (Takimoto et al., 2001). Both Sendai virus and NDV M proteins have been shown to physically associate with actin (Giuffre et al., 1982) and EM-based studies of measles virus and HRSV have revealed a tight association between actin filament formation and budding virions (Bohn et al., 1986; Jeffree et al., 2007).

Drug treatments that disrupt the cytoskeleton generally have deleterious effects on paramyxovirus replication. Multiple steps in the lifecycle of HRSV, for example, including viral entry and virus exit, are impaired upon disruption of the cytoskeleton, with severe disruptions in virus assembly and release following perturbation of either actin or microtubule networks (Burke et al., 1998; Kallewaard et al., 2005). Using molecular beacons to visually track RNPs, HRSV filament assembly and motion were recently examined in live cells, and myosin motor-driven transport on the actin cytoskeleton was postulated to drive the migration and/or rotational motion of the filaments (Santangelo and Bao, 2007). In the case of hPIV3, nocodazole-mediated disruption of microtubules significantly impairs the release of infectious virus from cells, although depolymerization of actin microfilaments with cytochalasin D had no effect on hPIV3 release (Bose et al., 2001). Microtubule disruption by nocodazole and colchicine treatments led to asymmetric budding of Sendai virus, similar to that observed with the pantropic F1-R mutant (Tashiro et al., 1993). Hence, efficient and directional budding of some paramyxoviruses depends on intact microtubules (Bose et al., 2001; Tashiro et al., 1993).

5.4 Trafficking of paramyxovirus M proteins through the cell nucleus

Although paramyxovirus replication takes place in the cytoplasm, various paramyxoviral proteins have been found to traffic to the nucleus during the course of infection (Coleman and Peeples, 1993; Ghildyal et al., 2009; Ghildyal et al., 2005a; Ghildyal et al., 2003; Peeples et al., 1992; Peeples, 1988; Rodriguez et al., 2004; Sato et al., 2006; Shaw et al., 2005; Watanabe et al., 1996; Yoshida et al., 1976). Early during infection, the M proteins of HRSV, NDV, and Sendai virus have all been observed in the nucleus, while later during infection they are localized mainly in the cytoplasm as well as associated with cell membranes (Ghildyal et al., 2003; Peeples et al., 1992; Peeples, 1988; Yoshida et al., 1976). As discussed earlier, altered Nipah virus M proteins with disruptions to the 62-YMYL-65 or 92-YPLGVG-97 sequences are retained in the nuclei of transfected cells, consistent with the possibility that Nipah virus M protein undergoes nuclear-cytoplasmic shuttling (Ciancanelli and Basler, 2006; Patch et al., 2008). By analogy with VSV, it has been suggested that transient nuclear localization of paramyxovirus M proteins in the early phase of infection could allow for M-mediated inhibition of cellular nuclear processes (Ghildyal et al., 2006). Transient nuclear localization of M proteins may also serve to delay the budding of particles until late times in infection, when sufficient quantities of viral glycoproteins and RNPs have accumulated. Functional nuclear localization signals (NLSs) of the NDV and HRSV M proteins have been characterized. The NLS of NDV M protein is a bipartite NLS composed of basic amino acid clusters (Coleman and Peeples, 1993). HRSV M protein is recruited into

the nucleus through direct recognition by a nuclear import receptor, importin beta 1 (Ghildyal et al., 2005a).

Late in paramyxovirus infection, large quantities of M protein are needed outside the nucleus at locations of virus assembly. In the case of HRSV M protein, nuclear export is mediated by a leucine-rich nuclear export signal and is dependent on Crm-1 (exportin-1) (Ghildyal et al., 2009). Recombinant virus harboring a mutated nuclear export signal within its M protein could not be recovered, and treatment of HRSV-infected cells with a Crm-1 inhibitor, leptomycin B, prevented nuclear export of HRSV M protein and impaired virus production (Ghildyal et al., 2009). These findings support a model in which paramyxovirus M protein nuclear localization is meant to be transient, and failure to exit from the nucleus impairs accumulation of the protein at virus assembly sites at levels required for virus budding.

6. Conclusions

Substantial progress has been made in recent years towards understanding both unique and shared processes used by paramyxoviruses and other enveloped viruses during virus particle formation. Roles for paramyxovirus M proteins, glycoproteins, nucleocapsid proteins, and accessory proteins during virus assembly have been clarified. Mechanisms allowing for directional budding of paramyxoviruses from polarized cells have been defined, and the importance of lipid raft microdomains as virus assembly platforms has been demonstrated. However, questions regarding important aspects of the paramyxovirus assembly process remain unresolved. For example, although paramyxoviruses clearly differ from one another in the fundamental components required for particle formation, the biological implications of these differences remain unclear. Structural information is still lacking for most paramyxovirus M proteins, and consequently the molecular determinants that drive viral protein-protein interactions during virus assembly remain poorly understood. While host factors clearly play important roles in the budding of many paramyxoviruses, specific mechanisms of host factor recruitment remain largely undefined. Future efforts in these and related areas will provide a clearer understanding of the events which must occur for virions to form, and may contribute to the development of effective antiviral strategies aimed at blocking the late steps of paramyxovirus lifecycles.

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Abbreviations used

AmotL1	angiomotin-like 1
ARE	apical recycling endosome
CDV	canine distemper virus
Crm1	exportin1
DN	dominant-negative
DRM	detergent-resistant membrane
ESCRT	endosomal sorting complex required for transport

F	fusion
G	glyco-
H	hemagglutinin (paramyxovirus)
HA	hemagglutinin (influenza virus)
HA-Ub	hemagglutinin-tagged ubiquitin
HMGCR	3-hydroxy-3-methylglutaryl-coenzyme A
HN	hemagglutinin-neuraminidase
hPIV	human parainfluenza virus
HRSV	human respiratory syncytial virus
HSP	heat shock protein
L	large
M	matrix
MVB	multivesicular body
MβCD	methyl-β-cyclodextrin
NA	neuraminidase
NDV	Newcastle disease virus
NLS	nuclear localization signal
N	nucleocapsid
NP	nucleocapsid
P	phospho-
PIV5	parainfluenza virus 5
Rab11-FIP	Rab11 family interacting proteins
RNP	ribonucleoprotein
SSPE	subacute sclerosing panencephalitis
VLP	virus-like particle
Vps	vacuolar protein sorting
VSV	vesicular stomatitis virus

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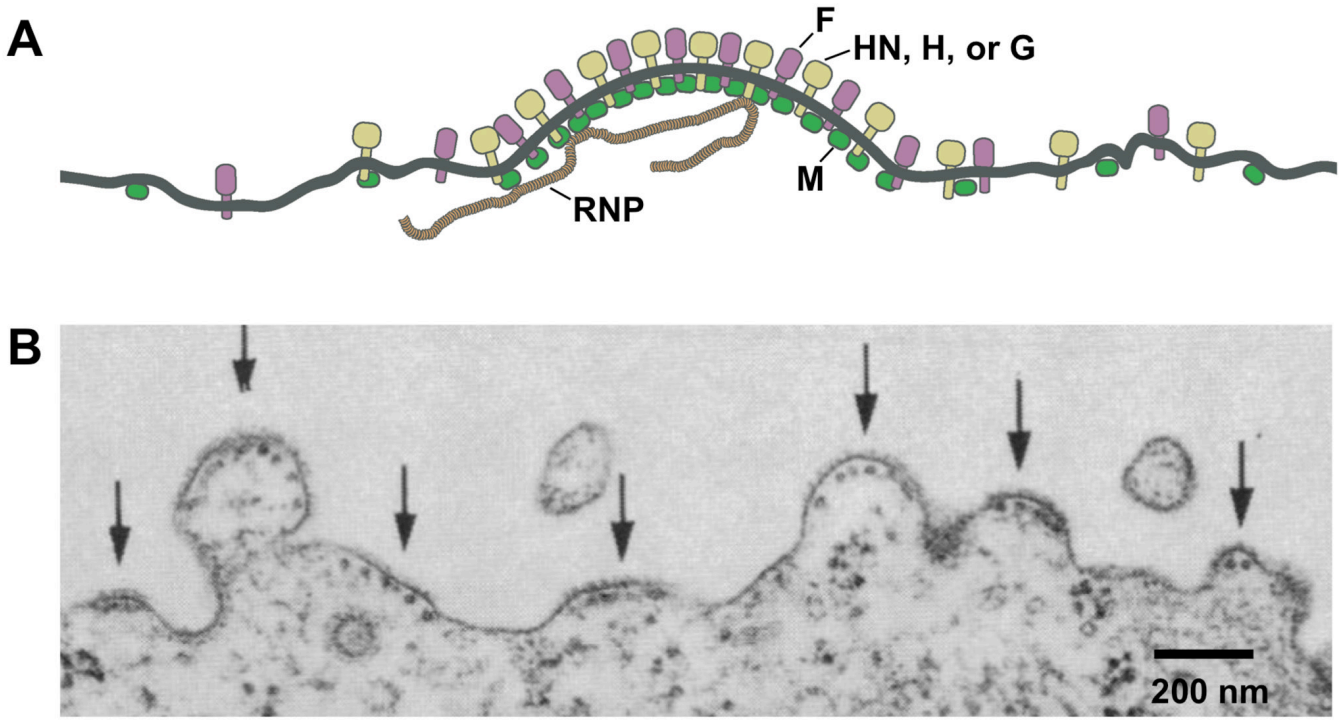

Figure 1. Budding of paramyxovirus particles

(A) Schematic illustration of paramyxovirus structural components that have assembled together on a cellular membrane, in preparation for budding. (B) Thin section of a Sendai virus-infected MDCK cell, visualized by electron microscopy. Arrows indicate individual sites from which virus budding has initiated on the apical cell surface. The nucleocapsid is observed aligning below the areas of curving membrane. (Adapted from Rodriguez-Boulan and Sabatini, 1978, with the author's permission).

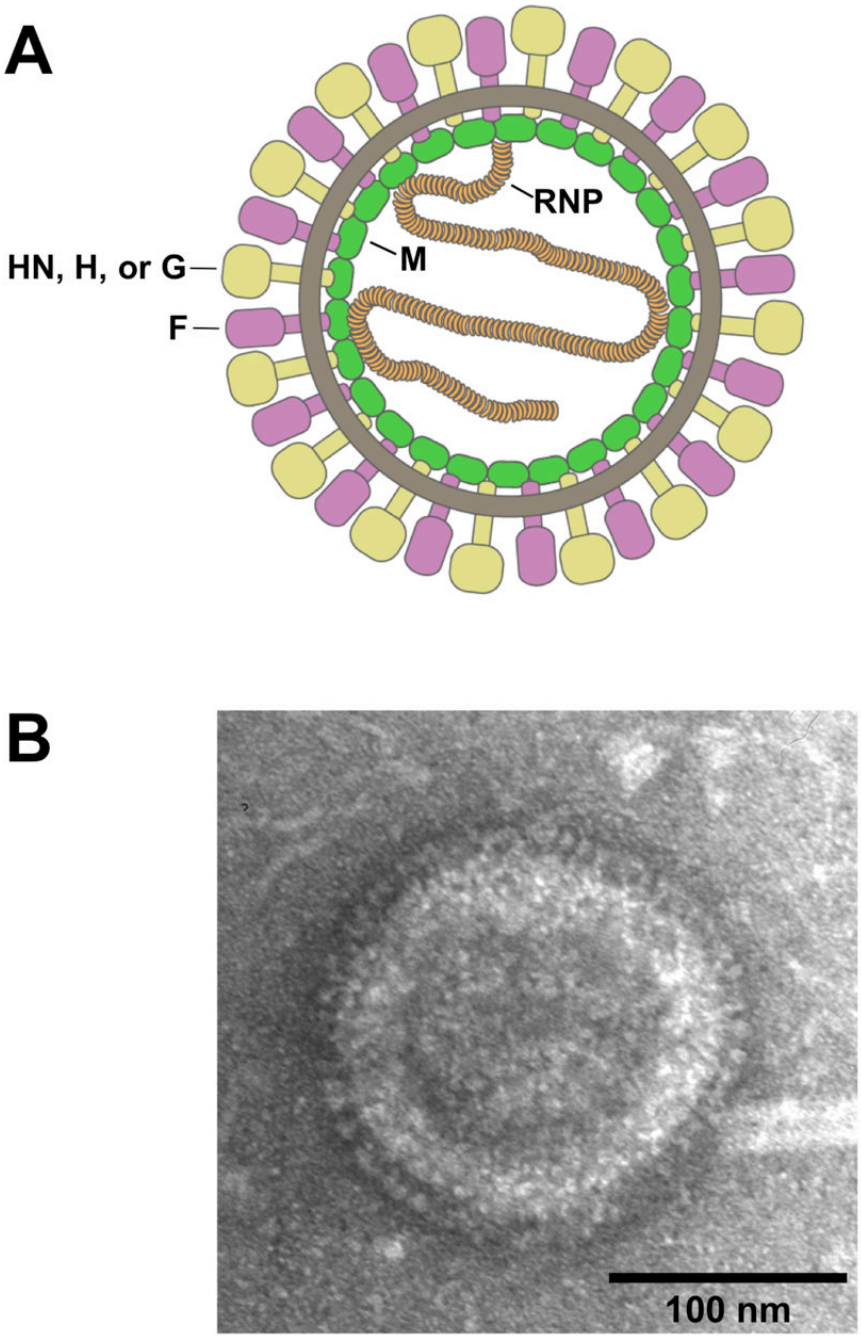

Figure 2. Paramyxovirus virions

(A) Schematic representation of a spherical paramyxovirus virion illustrating M protein lining the inner surface of the lipid envelope, the viral glycoproteins decorating the virion surface, and the helical RNP enclosed within. (B) A mumps virion purified by sucrose gradient centrifugation and imaged by transmission electron microscopy with negative staining. Glycoproteins are visible as a spike layer embedded in the envelope. (Adapted from Li et al., 2009, with the publisher's permission).

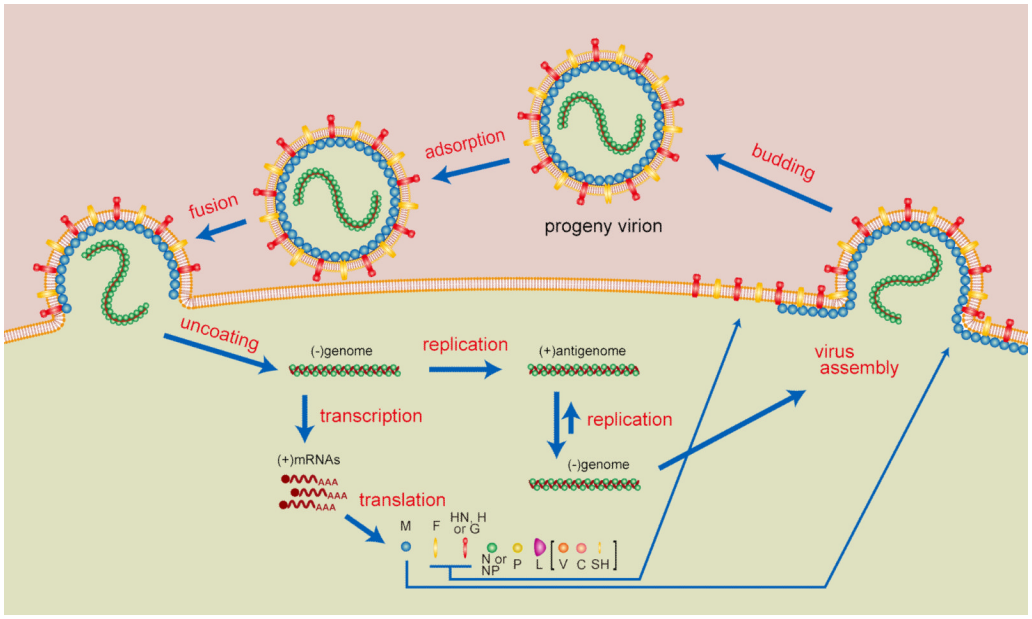

Figure 3. Schematic illustration of a paramyxovirus life cycle

For details, refer to the text. Viral transcription and genome replication occur in the cytoplasm. Following their synthesis, viral structural proteins and RNPs assemble together at the infected cell plasma membrane for budding. Additional processes mediated by the viral accessory proteins (shown surrounded by brackets) are not illustrated.

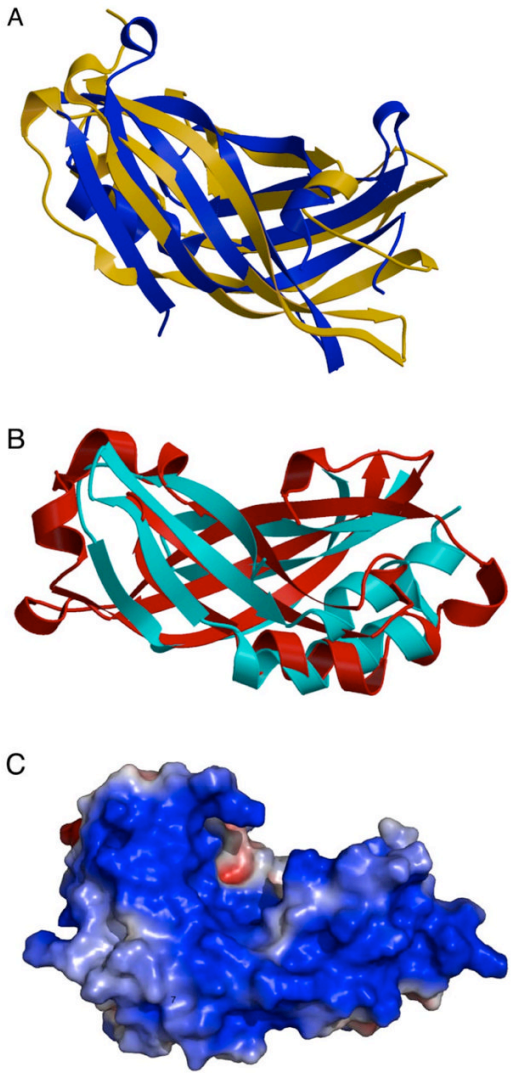

Figure 4. Atomic structure of HRSV M protein

(A) Ribbon diagrams illustrating the similar beta-sheet arrangements of the HRSV M protein (blue) and Ebola virus VP40 (yellow) N-terminal domains. (B) Ribbon diagrams illustrating the similar beta-sheet arrangements of the HRSV M protein (red) and Ebola virus VP40 (cyan) C-terminal domains. (C) Space filling model of the HRSV M protein structure, with electrostatic surface potential depicted in colors ranging from red to blue. An extensive positively-charged (blue) surface is shown. (HRSV M protein PDB code 2VQP, Ebola virus VP40 PDB code 1ES6; figure adapted from Money et al., 2009, with the author's permission).

Table 1

Differing requirements for paramyxovirus VLP production

Virus	Viral proteins needed for efficient VLP production	Notes	References
hPIV1	M	Nucleocapsid-like structures are incorporated into the VLPs when NP protein is expressed	Coronel et al., 1999
Sendai virus	M	F protein enhances VLP production, but HN protein does not; C protein enhances VLP production, likely through recruitment of the host protein Aip1/Alix	Takimoto et al., 2001; Sugahara et al., 2004
NDV	M	Glycoproteins and NP protein can be incorporated into VLPs, but do not enhance M-driven VLP production; incorporation of glycoproteins into VLPs becomes efficient when both glycoproteins are expressed	Pantua et al., 2006
PIV5	M, NP, and F; or M, NP, and HN	M protein expressed alone does not result in VLP production; F and HN proteins are necessary but redundant for VLP production	Schmitt et al., 2002
Mumps virus	M, NP, and F	M protein expressed alone results in very little VLP production; F protein strongly enhances VLP production, but HN protein does not	Li et al., 2009
Measles virus	M	F protein does not enhance M- driven VLP production	Pohl et al., 2007; Runkler et al., 2007
Nipah virus	M	Glycoproteins and N protein can be incorporated into VLPs when expressed, but do not enhance M-driven VLP production	Ciancanelli and Basler, 2006; Patch et al., 2007